α-N-ACETYLGALACTOSAMINIDASE FROM *CLOSTRIDIUM PERFRINGENS*

CROSSREFERENCE TO RELATED APPLICATIONS

This application is a Continuation application of United States Serial Number 09/185,476, filed November 3, 1998, which claims benefit under 35 USC §119(e) of United States Provisional Application Serial Number 60/064,683, filed November 3, 1997.

BACKGROUND OF THE INVENTION

1. FIELD OF THE INVENTION

The present invention relates to an improved method of isolating a purified α-N-acetylgalactosaminidase from *Clostridium perfringens* to be used in the conversion of erythrocytes to type O cells to render the cells useful for transfusion therapy.

2. DESCRIPTION OF RELATED ART

The A, B, and H antigens are a clinically significant blood group (Landsteiner, 1901; Mollison et al, 1987). These antigens are terminal immunodominant monosaccharides on erythrocyte membrane glycoconjugates (Harmening, 1989). High densities of these epitopes are present on erythrocyte membranes and antibodies bound to these antigens

readily fix complement (Economidou et al., 1967; Romano and Mollison, 1987). Because these epitopes are ubiquitous in nature, immuno-potent and naturally occurring, complement fixing antibodies occur in individuals lacking these antigens, and transfusion of incompatible blood results in fatal hemolytic transfusion reactions (Fong et al., 1974; Schmidt, 1980).

Complex sugar chains in glycolipids and glycoproteins have often been implicated in the growth and development of eukaryotes (Wantanabe et al., 1976). In particular, complex sugar chains play an important part in the recognition of self in the immune system (Mollison, 1987). Glycosidases (both exoglycosidases and endoglycosidases) are enzymes which can modify carbohydrate membrane epitopes, thereby modulating the immune response (Goldstein et al., 1982).

United States patents 4,330,619, issued May 18, 1982; 4,427,777, issued January 24, 1984; and 4,609,627, issued September 2, 1986, all to Goldstein, relate to the enzymatic conversion of certain erythrocytes to type O erythrocytes. Since type O erythrocytes can be safely transfused into type A, type B, type A,B recipients, as well as O recipients, type O erythrocytes have significant value in transfusion therapy. The above-mentioned United States patent 4,609,627 discloses the conversion of certain sub-type A and A,B erythrocytes to type O erythrocytes utilizing an α-N-acetylgalactosaminidase fraction from fresh chicken livers. The patent also discusses the significant potential of such enzymes to be used in the conversion of type A_2 erythrocytes to type O erythrocytes or type A_2 erythrocytes to type B erythrocytes.

The α-N-acetylgalactosaminidase from domestic chickens is such an enzyme as described above which degrades the human blood group A

epitope (Hata et al., 1992). Degradation of the blood group A antigen produces the H antigen, also known as blood group O. Blood group O red blood cells are generally universally transfusable within the ABO blood group system.

The enzyme α-N-acetylgalactosaminidase [EC 3.2.1.49] is a class of exoglycosidases that have been purified from both procaryotes and eucaryotes (McGuire et al., 1972; McDonald et al., 1972; Kadowaki et al., 1989; Itoh and Uda, 1984; Nakagawa et al., 1987; Kubo, 1989; Weissman et al., 1969; Weissman, 1972). Despite the use of this enzyme because of its ready availability from the livers of domesticated chickens, the use is limited. This is based on the lack of published reports regarding the use of commercially useful purification methods for making preparations having no detectable protease or other glycosidase activities along with proven homogeneity (Goldstein, 1984). The low pH optimum of chicken liver enzyme makes it less useful because large masses of enzyme are required. Additionally, red cells must be extensively washed to lower the pH so that the enzyme can efficiently convert red cells from type A to type O or type A_2B to type B. Similar problems exist with preparations from other sources including *Clostridium perfringens* (McGuire et al, 1972). Critically, without a commercially viable method to provide enzymatic activity free of extraneous proteases, neuraminidsae, and glycosidases, there is limited commercial value since the use of a nonhomogeneous enzyme preparation has the potential to damage erythrocyte membranes, therefore leading to poor *in vivo* viability. It would be particularly advantageous to be able to a isolate α-N-acetylgalactosaminidase from *Clostridium perfringens* free of contaminants,

particularly neuraminidase, while also having additional properties.

It would therefore be useful to develop an α-N-acetylgalactosaminidase which is homogeneous and capable of enzymatic activity against the blood group A epitope.

SUMMARY OF THE INVENTION

According to the present invention, an isolated and purified α-N-acetyl-D-galactosaminidase from *Clostridium perfringens* is disclosed. A method for purifiying and isolating the α-N-acetyl-D-galactosaminidase from *Clostridium perfringens* by removing neuramidases is disclosed. A process for using the α-N-acetyl-D-galactosaminidase from *Clostridium perfringens* in altering erythrocytes to type O erythrocytes is disclosed. A process for altering cells expressing blood group A epitope by using α-N-acetyl-D-galactosaminidase isolated from *Clostridium perfringens* in altering the cells expressing blood group A epitope to cells expressing blood group O epitope is disclosed.

DESCRIPTION OF THE DRAWINGS

Other advantages of the present invention are readily appreciated as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings wherein:

Figure 1 is a bar graph showing the optimal expression occurred with Brain Heart Infusion (BHI), also shown in the Todd-Hewitt media used by Levy and Aminoff and the Difco brand BHI media;

Figure 2 shows enzyme expression as a function of BHI concentration, optimal concentrations ranging from 20 to 80 g/l;

Figure 3 shows a set of time courses showing BHI from Difco induced the highest expression;

Figure 4 shows K_2HPO_4 having an optimal range of concentration between 4 and 8 g/l;

Figure 5 shows a cysteine concentration curve. Useful concentrations ranging from 0.05 to 0.4 g/l, concentration of 0.05 g/l seemed adequate for enzyme expression;

Figure 6 is a glucose concentration curve; Lower glucose concentrations enhancing expression, a concentration range from 0 to 0.1 g/l being optimal;

Figure 7 shows a porcine mucin concentration curve; higher mucin concentrations enhancing enzyme expression, a concentration greater than 4 g/l showing optimal expression;

Figure 8 shows an SDS-PAGE with lane 1 being the unreduced α-N-acetylgalactosaminidase, lane 2 being the reduced α-N-acetylgalactosaminidase and lane 3 being the molecular weight standards (97.4, 66.2, 45.0, 31.0, 21.5, and 14.3 kDa);

Figure 9 shows a gel filtration wherein α-N-acetylgalactosaminidase is indicated by the arrow;

Figure 10 shows a Hydroxyapatite HPLC wherein activity is shown with of U/ml being a function of fraction number;

Figure 11 shows an SDS-PAGE of column fractions with a zinc stain, lane 1 being the molecular weight standards (97.4, 66.2, 45.0, 31.0, 21.5, and 14.3 kDa) and lanes 2 through 6 showing the column fractions #48 to #52;

Figure 12 shows enzyme activity as a function of pH, measurements being performed as described in the Materials and Methods sections herein, all data points being the mean of three independent duplicate determinations;

Figure 13 shows enzyme activity as a function of ionic strength, measurements being performed as described in the Materials and Methods sections herein, all data points being the mean of three independent duplicate determinations;

Figure 14 shows the degradation of the A_2 epitope as a function of enzyme concentration; Δ O.D.410 measuring the hydrolysis of the terminal N-acetyl-α-D-galactosamine from the blood group A_2 epitope, all data points being the mean of duplicate independent determinations; and

Figure 15 shows the N-terminal sequence of α-N-acetylgalactosaminidase.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of producing a purified α-N-acetylgalactosaminidase (SEQ ID No:1-16 and Figure 15), and functional analogs thereof, from *Clostridium perfringens* which are free of neuraminidase activity. A detailed description of the isolation protocols and its properties are set forth in the Experimental Section.

By functional analogs, it is meant that an analog is generally at least

70% homologous over any portion that is functionally relevant. In more preferred embodiments the homology is at least 80% and can approach 95% homology to the α-N-acetylgalactosaminidase. The amino acid sequence of an analog can differ from that of the α-N-acetylgalactosaminidase when at least one residue is deleted, inserted or substituted. Differences in glycosylation can provide analogs. The molecular weight of the α-N-acetylgalactosaminidase can vary between the analog and the present invention due to carbohydrate differences. For example, the following are the N-terminal and tryptic amino acid sequence data obtained from the purified enzyme. These sequences (SEQ ID Nos: 8-15) have a percent homology of at least 90%.

N-terminal sequence: (K)VLGNYIQRNFHYDGKSFYT(T)(S)FLN (SEQ ID No:8).

Internal Sequence (trypsin fragments):

EDGSVEVK (SEQ ID No:9);
ATVSLPR (SEQ ID No:10);
LPAA(F/I)RKA (SEQ ID No:11);
IIILKEF (SEQ ID No:12);
DSQYYEFLIER (SEQ ID No:13);
(K)YDVVLGNK (SEQ ID No:14); and
(F)PNELK (SEQ ID No:15).

CNBr Cleavage: ANFNGYYVELGQPIYAKSL (SEQ ID NO.: 16)

The *C. perfringens* orf 325 sequence is found at Table 2. The CPE0325 coding sequence starts at the ATG start codon at nucleotide 108014 and extends to the TAA stop codon at nucleotide 109903. This DNA sequence is part of GenBank accession number AP003186, which is section 2 of the complete genome sequence of *Clostridium perfringens* strain 13. The CPE0325 coding sequence is annotated as a hypothetical protein and no

functional assignment for this protein is included in the annotation that accompanies this GenBank entry. Query of the complete genome sequence of *Clostridium perfringens* with the empirically determined N-terminal amino acid sequence of purified alpha-N-acetylgalactosaminidase resulted in a match (100% sequence identity) between the 25 residue peptide sequence and amino acids 2-26 of CPE0325. The 25 amino acid sequence was not present in any other deduced *C. perfringens* translation product (protein), nor any other protein sequence in the GenBank database. Furthermore, each of seven internal peptide sequences (determined by amino acid sequencing of tryptic peptides) can be identified within the CPE0325 ORF. These findings identify the CPE0325 hypothetical protein as the alpha-N-acetylgalactosaminidase of *C. perfringens*.

More specifically, the N-terminal sequence set forth above follows the Met start codon. The internal fragments are scattered. Sequence ID Numbers 9, 13, and 15 are in the last 40 residues. Sequence ID Number 10 is within the first 10 residues, Sequence ID Numbers 11 and 12 overlap and the best matches are at about residue 210, Sequence ID Number 14 is at about 455.

Standard molecular biology techniques known in the art and not specifically described are generally followed as in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New York (1989, 1992), and in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1989). Additionally, standard methods in immunology known in the art and not specifically described are generally followed as in Stites et al. (eds), *Basic and Clinical Immunology (8th*

Edition), Appleton & Lange, Norwalk, CT (1994) and Mishell and Shiigi (eds), *Selected Methods in Cellular Immunology*, W.H. Freeman and Co., New York (1980).

Cloning techniques are provided by the present invention. In general, recombinant α-N-acetylgalactosaminidase are superior to the native lysosomal enzymes for these applications.

Immunoassays are also provided by the present invention. In general, ELISAs are the preferred immunoassays employed to assess a specimen. Both polyclonal and moloclonal antibodies can be used in the assays. The specific assay to be used can be determined by one skilled in the art.

Antibody production is provided by the present invention. Antibodies can be prepared against the immunogen, or any portion thereof, for example a synthetic peptide based on the sequence. As stated above, antibodies are used in assays and are therefore used in determining if the appropriate enzyme has been isolated. Antibodies can also be used for removing enzymes from red cell suspensions after enzymatic conversion. Immunogens can be used to produce antibodies by standard antibody production technology well known to those skilled in the art as described generally in Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Springs Harbor Laboratory, Cold Spring Harbor, NY, 1988 and Borrebaeck, *Antibody Engineering - A Practical Guide*, W.H. Freeman and Co., 1992. Antibody fragments can also be prepared from the antibodies and include Fab, $F(ab')_2$, and Fv by methods known to those skilled in the art.

The enzyme obtained by the method of the present invention is characterized by the following:

1. Homogeneous by SDS-PAGE;

2. Molecular weight by SDS-PAGE of approximately 72.1kDa;

3. Molecular weight by molecular sieve chromatography of approximately 57.5 kDa;

4. Specific activity of approximately 40.54 U mg^{-1} min^{-1} using 1mM PNP-N-acetylyl-a-D-galactosaminide as a substrate and BSA as a protein standard in a BioRad Protein assay;

5. An approximate pH optimum of 6.5 to 7.0;

6. Undetectable neuraminidase activity in the tested assay systems;

7. Low to undetectable protease activity; and

8. Activity against the blood group A_2 on erythrocyte membranes using an ELISA assay.

These properties make the enzyme isolated and purified by the method of the present invention useful in enzymatic conversion technology to produce type O red blood cells.

Briefly the method for isolating and purifying the enzyme begins by expressing the enzyme in stationary cultures. After 92 hours the medium is harvested by centrifugation. The mineral oil overlay is separated from the medium in a separatory funnel and the enzyme purified from the cell free expired medium.

More specifically, the procedures of *Clostridium perfringens* α-N-acetylgalactosaminidase purification generally include the following steps: 1)obtaining a *Clostridium perfringens* culture; 2) conducting a $(NH_4)_2SO_4$ precipitation; 3) pass the preparation through a S-200 (I) column; 4) load the preparation into a DEAE Sephadex A-50 (I) column; 5) load the preparation

into a PBE 94 exchanger; 6) load the preparation into a SP Sephadex C-50 column; 7) load the preparation into a DEAE Sephadex A-50 (II) column; and 8) load the preparation into a Hydroxyapatite Type I column.

Various buffers can be used for the purification. Initially, a cell culture is cultured, as previously described (Levy and Aminoff, 1980) with a modification. It was found that these methods are not beneficial because little or no enzymatic activity was observed using the ATCC strain and media conditions described by those authors. Therefore, Brain Heart Infusion (BHI) is substituted for Todd-Hewitt broth because it was found that BHI provides optimal expression as is shown in Figure 1. The BHI media contains BHI, K_2HPO_4, cysteine, glucose, and porcine gastric mucin. Optimal concentration of the components of the BHI media are as follows: BHI concentration was optimal in the range of 20 to 80 g/l (Figure 2) further, it was found that BHI from Difco induced the greatest expression (Figure 3); K_2HPO_4 is added to the media to maintain pH with a useful range of 4 to 8 g/l (Figure 4); cysteine has useful concentrations in the range of 0.05 to 0.4 g/l (Figure 5); lower glucose concentrations enhanced expression and a concentration range from 0 to 0.1 g/l is optimal (Figure 6); and higher porcine mucin concentration enhanced enzyme expression with concentrations higher than 4 g/l being optimal (Figure 7).

The cell culture is separated from the mineral oil and centrifuged. The cell-free supernatant is brought to 70% saturation with solid $(NH_4)_2SO_4$, and is stirred gently at 4° C for two hours. The precipitate is collected by centrifugation and then dissolved in 1/50th of the starting volume in 50 mM Na acetate buffer, pH 5.0, containing 1.0 mM DTT, 0.1% (v/v) Tween 80 and 0.01% (w/v) NaN_3. The suspension is then centrifuged to collect the

precipitate-free supernatant.

The supernatant is then applied to a column of Sephacryl S-200 which is equilibrated in a Na acetate buffer. The column is developed with equilibration buffer at 4°C. Fractions are obtained which have enzyme activity and are then pooled and dialyzed against a buffer.

This dialyzed pool is applied to a column of DEAE Sephadex A-50 which is equilibrated in a Tris-HCl buffer at 4°C. Again fractions containing enzyme activity are pooled and dialyzed against a buffer.

The dialyzed pool is then applied to a column of PBE 94 chromatofocusing resin which is equilibrated. Elution is accomplished by developing the column with Polybuffer 74 solution. Fractions containing enzyme activity are pooled and dialyzed against a sodium acetate buffer.

Then the dialyzed preparation is applied to a column of SP Sephadex C-50 which is equilibrated in a sodium acetate buffer. The effluent is collected and retained upon which time the column is washed with sodium acetate buffer. The effluent and wash are combined and dialyzed against a Tris-HCl buffer.

Next, the dialyzed pool is applied to a column of DEAE Sephadex A-50 which is equilibrated in a Tris-HCl buffer. Fractions containing enzyme activity are pooled. This enzyme pool was dialyzed and a K_2HPO_4 buffer and then applied to a BioScale Ceramic Hydroxyapatite, Type I column for HPLC. Fractions obtained from this are retained and pooled and then stored at 4°C.

Protein concentrations are quantitated with the Bio-Rad protein assay, or any assay known by one skilled in the art which quantitates protein concentrations. Enzymatic activity is determined by measuring the production of *p*-nitrophenol (PNP) from PNP-N-acetyl-α-D-galactosaminide.

Neuraminidase activity is then measured using 4-MU-α-N-acetylneuraminic acid by an adaptation of the method of Dean et al. (1977).

The above discussion provides a factual basis for the use of α-N-acetyl-D-galactosaminidase. The methods used with and the utility of the present invention can be shown by the following non-limiting examples and accompanying figures.

The following Experimental section provides a specific extraction process and analytical procedure characterizing the derived purified enzyme.

EXAMPLES

General Methods:

General methods in molecular biology: Standard molecular biology techniques known in the art and not specifically described are generally followed as in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New York (1989, 1992), and in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1989). Polymerase chain reaction (PCR) is carried out generally as in *PCR Protocols: A Guide To Methods And Applications*, Academic Press, San Diego, CA (1990). Reactions and manipulations involving other nucleic acid techniques, unless stated otherwise, are performed as generally described in Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, and methodology as set forth in United States patents 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057 and incorporated herein by reference. In-situ (In-cell) PCR in combination

with Flow Cytometry can be used for detection of cells containing specific DNA and mRNA sequences (Testoni et al, 1996, Blood 87:3822.) Additionally, cloning is carried out as generally described in *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press.

General methods in immunology: Standard methods in immunology known in the art and not specifically described are generally followed as in Stites et al.(eds), Basic and Clinical Immunology (8th Edition), Appleton & Lange, Norwalk, CT (1994) and Mishell and Shiigi (eds), Selected Methods in Cellular Immunology, W.H. Freeman and Co., New York (1980).

Immunoassays

In general, ELISAs are the preferred immunoassays employed to assess a specimen. ELISA assays are well known to those skilled in the art. Both polyclonal and monoclonal antibodies can be used in the assays. Where appropriate other immunoassays, such as radioimmunoassays (RIA) can be used as are known to those in the art. Available immunoassays are extensively described in the patent and scientific literature. See, for example, United States patents 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521 as well as Sambrook et al, *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor, New York, 1989

Antibody Production

Antibody Production: Antibodies can be either monoclonal, polyclonal or recombinant. Conveniently, the antibodies can be prepared against the immunogen or portion thereof for example a synthetic peptide based on the sequence, or prepared recombinantly by cloning techniques or the natural gene product and/or portions thereof can be isolated and used as the immunogen. Immunogens can be used to produce antibodies by standard antibody production technology well known to those skilled in the art as described generally in Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988 and Borrebaeck, *Antibody Engineering - A Practical Guide*, W.H. Freeman and Co., 1992. Antibody fragments can also be prepared from the antibodies and include Fab, $F(ab')_2$, and Fv by methods known to those skilled in the art.

For producing polyclonal antibodies a host, such as a rabbit or goat, is immunized with the immunogen or immunogen fragment, generally with an adjuvant and, if necessary, coupled to a carrier; antibodies to the immunogen are collected from the sera. Further, the polyclonal antibody can be absorbed such that it is monospecific. That is, the sera can be absorbed against related immunogens so that no cross-reactive antibodies remain in the sera rendering it monospecific.

For producing monoclonal antibodies the technique involves hyperimmunization of an appropriate donor with the immunogen, generally a mouse, and isolation of splenic antibody producing cells. These cells are fused to a cell having immortality, such as a myeloma cell, to provide a fused cell hybrid which has immortality and secretes the required antibody. The cells are then cultured, in bulk, and the monoclonal antibodies harvested from the culture media for use.

For producing recombinant antibody (see generally Huston et al, 1991; Johnson and Bird, 1991; Mernaugh and Mernaugh, 1995), messenger RNAs from antibody producing B-lymphocytes of animals, or hybridoma are reverse-transcribed to obtain complimentary DNAs (CDNAs). Antibody cDNA, which can be full or partial length, is amplified and cloned into a phage or a plasmid. The cDNA can be a partial length of heavy and light chain cDNA, separated or connected by a linker. The antibody, or antibody fragment, is expressed using a suitable expression system to obtain recombinant antibody. Antibody cDNA can also be obtained by screening pertinent expression libraries.

The antibody can be bound to a solid support substrate or conjugated with a detectable moiety or be both bound and conjugated as is well known in the art. (For a general discussion of conjugation of fluorescent or enzymatic moieties see Johnstone & Thorpe, *Immunochemistry in Practice*, Blackwell Scientific Publications, Oxford, 1982.) The binding of antibodies to a solid support substrate is also well known in the art. (see for a general discussion Harlow & Lane *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Publications, New York, 1988 and Borrebaeck, *Antibody Engineering - A Practical Guide*, W.H. Freeman and Co., 1992) The detectable moieties contemplated with the present invention can include, but are not limited to, fluorescent, metallic, enzymatic and radioactive markers such as biotin, gold, ferritin, alkaline phosphatase, b-galactosidase, peroxidase, urease, fluorescein, rhodamine, tritium, ^{14}C and iodination.

Recombinant Protein Purification

Marshak et al, "Strategies for Protein Purification and Characterization. A

laboratory course manual." CSHL Press, 1996.

Example 1:

The following is the detailed description of the protocol of the present invention for isolating a purified α-N-acetylgalactosaminidase from *Clostridium perfringens* free of neuraminidase activity to be used in the conversion of erythrocytes to type O cells to render the cells useful for transfusion therapy. The properties of the isolated enzyme are also provided.

METHODS AND MATERIALS:

Materials

Clostridium perfringens was obtained from the American Type Culture Collection (ATCC), Rockville, MA. Media were purchased from Difco Laboratories, Detroit, MI. Protein assay dye reagent was obtained from Bio-Rad, Hercules, CA. An anaerobic system and BBL GasPak Plus were purchased from Becton Dickinson and Company, Cockeysville, MD. SDS polyacrylamide gels and HPLC (high pressure liquid chromatography) columns were purchased from Bio-Rad, Hercules, CA. Dithiothreitol (DTT) and K_2HPO_4 were acquired from Fisher Biotech, Pittsburgh, PA. Porcine gastric mucin, L-cysteine, glucose, bovine serum albumin (BSA), PolyBuffer 74, substrates (4-methylumbel-liferyl-α-D-N-acetylneuraminic acid, PNP- and ONP-N-acetyl-α-D-galactosaminide), all other chemicals, and chromatography resins (Sephacryl S-200, DEAE Sephadex A-50, PBE 94, and SP Sephadex C-50) were obtained from Sigma Chemical Company, St. Louis, MO. Endoprotease substrates were purchased from Boehringer

Methods

Bacterial Culture: *Clostridium perfringens* was cultured as previously described (Levy and Aminoff 1980), however, Brain Heart Infusion (BHI) was substituted for Todd-Hewitt broth in the formulation. A lyophilized vial of *Clostridium perfringens* strain ATCC 10543 was suspended in 1.0 ml of prewarmed and prereduced BHI media containing 40 g/l BHI, 8 g/l K_2HPO_4, 0.05 g/l cysteine, 1 g/l glucose and 5 g/l porcine gastric mucin. The suspension was added to 10 ml of warm reduced media and incubated at 37°C. After ten to twelve 24-hour passages, 10 ml of culture was added to 2.5 liters of warmed, reduced media overlaid with 400 ml of mineral oil. Pick up 1 ml aliquot from the culture flask and assay the α-N-acetylgalactosaminidase activity every 24 hours during the incubation. Plot the graph of the α-N-acetylgalactosaminidase activity v.s. time to determine the maximal enzyme activity. The mineral oil maintained an anaerobic environment. The bacteria were grown in standing culture at 37°C for approximately 72 to 96 hours. Enzyme production was monitored, and the culture was harvested after plateauing.

Purification of α-N-acetylgalactosaminidase:

Step 1. The culture was separated from mineral oil with a separatory funnel, and was centrifuged at 5,620 X g for 30 min. at 4°C. The cell-free supernatant was brought to 70% saturation with solid $(NH_4)_2SO_4$, and was then stirred gently at 4°C for 2 hours. The precipitate was collected by

centrifugation (5,620 X g, 2 hours, 4°C), and was dissolved in 1/50th of the starting volume in 50 mM Na acetate buffer, pH 5.0, containing 1.0 mM DTT, 0.1%(v/v) Tween 80 and 0.01%(w/v) NaN_3. The suspension was then centrifuged at 5,620 X g for 60 min. at 4°C to collect the precipitate-free supernatant.

Step 2. The supernatant was applied to a 5 x 90 cm column of Sephacryl S-200 equilibrated in 50 mM Na acetate buffer, pH 5.0, containing 1.0 mM DTT, 0.1% Tween 80 and 0.01% NaN_3, and the column was developed with equilibration buffer at 4°C. Fractions containing enzyme activity were pooled and dialyzed against 10 mM Tris-HC1 buffer, pH 8.0, containing 1.0 mM DTT, 0.1% Tween 80 and 0.01% NaN_3 at 4°C.

Step 3. The dialyzed pool was applied to a 1.5 x 20 cm column of DEAE Sephadex A-50 equilibrated in 10 mM Tris-HC1 buffer, pH 8.0, containing 1.0 mM DTT, 0.1% Tween 80 and 0.01% NaN_3 at 4°C. The elution was achieved by developing the column with a 200 ml linear gradient of 10mM Tris-HC1 buffer, pH 8.0, containing 0 to 500 mM NaC1, 1.0 mM DTT, 0.1% Tween 80 and 0.01% NaN_3 at 4°C. Fractions containing enzyme activity were pooled and dialyzed against 20 mM MES buffer, pH 6.2, containing 1.0 mM DTT, 0.1% Tween 80 and 0.01% NaN_3 at 4°C.

Step 4. After dialysis, the pool was applied to a 1 x 30 cm column of PBE 94 chromatofocusing, equilibrated in 20 mM MES buffer, pH 6.2, containing 1.0 mM DTT, 0.1% Tween 80 and 0.01% NaN_3 at 4°C. Elution was accomplished by developing the column with Polybuffer 74 solution, pH 3.8 at 4°C. Fractions containing enzyme activity were pooled and dialyzed against 10 mM Na acetate buffer, pH 5.0, containing 1.0 mM DTT, 0.1%

Tween 80 and 0.01% NaN_3 at 4°C.

Step 5. The dialyzed preparation was applied to a 1 x 5 cm column of SP Sephadex C-50 equilibrated in 10 mM Na acetate buffer, pH 5.0, containing 1.0 mM DTT, 0.1% Tween 80 and 0.01% NaN_3 at 4°C. The effluent was collected and retained, and the column was washed with 5 ml of 10 mM Na acetate buffer, pH 5.0, containing 1.0 mM DTT, 0.1% Tween 80 and 0.01% NaN_3. The effluent and wash were combined and dialyzed against 10 mM Tris-HC1, pH 8.0, containing 1.0 mM DTT, 0.1% Tween 80 and 0.01% NaN_3 at 4°C.

Step 6. The dialyzed pool was applied to a 1.5 x 5 cm column of DEAE Sephadex A-50 equilibrated in 10 mM Tris-HC1 buffer, pH 8.0, containing 1.0 mM DTT, 0.1% Tween 80 and 0.01% NaN_3 at 4°C. The elution was achieved by developing the column with a 100 ml linear gradient of 10 mM Tris-HC1 buffer, pH 8.0, containing 0 to 500 mM NaC1, 1.0 MM DTT, 0.1% Tween 80 and 0.01% NaN_3 at 4°C. Fractions with enzyme activity were pooled.

Step 7. The enzyme pool was dialyzed against 10 mM K_2HPO_4 buffer, pH 6.8, containing 1.0 mM DTT, 0.1% Tween 80 and 0.01% NaN_3 at 4°C, and applied to a Bio-Scale Ceramic Hydroxyapatite, Type 1 column for HPLC at room temperature. The enzyme was eluted with a continuous phosphate gradient of 10 to 400 mM K_2HPO_4. Fractions containing enzyme activity were pooled and stored at 4°C.

Characterization of α-N-acetylgalactosaminidases:

Protein concentrations were quantitated with the Bio-Rad protein assay

using BSA as a standard. Enzymatic activity was determined by measuring production of *p*-nitrophenol (PNP) from PNP-N-acetyl-α-D-galactosaminide. Enzyme aliquots were incubated in 200 μL of 40 mM NaH_2PO_4 buffer, pH 6.5, containing 1.0 mg mL^{-1} BSA, 1.0 mM DTT, and 1.0 mM PNP-N-acetyl-α-D-galactosaminide, at 37°C. The reactions were quenched with 1.0 mL of 0.25 M Na_2CO_3 and the OD 405 nm was measured. One unit of activity was defined as 1.0 μmole of substrate hydrolyzed per minute. Neuraminidase activity was measured using 4-MU-α-N-acetylneuraminic acid by an adaptation of the method of Dean et al. (1977).

The pH optimum was determined by incubating enzyme aliquots in 20 mM NaH_2PO_4 and 20 mM Na citrate, pH 2.0 to 8.0, containing, 1.0 mg mL^{-1} BSA, 1.0 mM DTT and 2.5 mM PNP-N-acetyl-α-D-galactosaminide at 37°C. The ionic strength optimum was determined by incubating aliquots of the enzyme in 2.5 mM NaH_2PO_4 buffer, pH 7.0, containing 1.0 mg mL^{-1} BSA, 2.5 mM PNP-N-acetyl-α-D-galactosaminide and 0 to 2.0 M NaCl at 37°C. The K_ms for ONP- and PNP-N-acetyl-α-D-galactosaminide were determined by varying the substrate concentration in 50 mM NaH_2PO_4 buffer, pH 7.0, containing 1.0 mg mL^{-1} BSA and 0.01% NaN_3, at 37°C. The native molecular weight was determined by applying a concentrated portion of the enzyme to a 1.5 x 40 cm column of Sephacryl S-200 equilibrated in 10 mM Tris-HC1 buffer, pH 8.0, containing 150 mM NaC1, 1.0 mM DTT, 0.1% Tween 80 and 0.01% NaN_3. Enzyme activity was measured as a function of fraction number, and compared to native molecular weight standards. SDS (sodium dodecyl sulfate) polyacrylamide electrophoresis (SDS-PAGE) was performed by a method of Laemmli (1970), and the protein bands were developed with

either Coomassie R-250 or zinc stain (Fernandez-Patron et al, 1992). Amino acid composition was analyzed on a Perkin Elmes (PE) ABD model 120A PTH Amino Acid Analyzer using UV detection. Aminopeptidase assays were performed by incubating aliquots of enzyme with 2.5 mM of various *p*-nitroaniline (PNA) substrates in 50 mM Na acetate, pH 6.0, containing 50 mM NaH_2PO_4, 1.0 mg mL^{-1} BSA, and 0.01% NaN_3, at 37°C (Macfarlane et al, 1988). The reactions were quenched with 1.0 ml of 0.25 M Na_2CO_3, and the OD at 405 nm was determined. One unit of aminopeptidase activity is defined as 1.0 μmole of PNA hydrolyzed per minute. Endoprotease assays were performed by an adaptation of the resorufin-labeled casein assay of Twining (1984). Blood group activity was determined with an enzyme linked immunosorbent assay (ELISA) as described by Hobbs et al. (1993).

Results

As shown in Table I, rapid purification of the enzyme was achieved with acceptable recoveries. Neuraminidase was the most undesirable contaminant that was efficiently removed by this purification scheme. The DEAE Sephadex A-50, PBE 94 chromatofocusing, and SP Sephadex C-50 columns removed the bulk of contaminating protein and neuramidase. Final purification was achieved by high pressure liquid chromotography (HPLC) on a Bio-Scale Ceramic Hydroxyapatite, Type I column. The specific activity of the purified enzyme ranged from 30.40 to 50.68 units milligram $protein^{-1}$ $minute^{-1}$ (X = 42.19, s.d. = 10.53, n = 3) with 1.0 mM PNP-N-acetyl-α-D-galactosaminide as the substrate. There was a 137 fold purification with an average recovery of 1.97%, Table 1. The enzyme was stable at 4°C for over

a year with less than 10% loss of activity.

The purified preparation had a single detectable band when analyzed by Coomassie R-250 staining of a 12% SDS PAGE. The mean molecular weight, as determined by SDS-PAGE under reducing conditions, was 72.1 kDa (s.d. = 1.1, n = 6) as illustrated in Figure 8. The mean native molecular weight was 57.5 kDa (s.d. = 3.2, n = 3) as calculated by gel filtration on Sephacryl S-200, Figure 9. SDS-PAGE and enzymatic activity in the peak Hydroxyapitite HPLC fractions correlated with the staining intensity of the 72.1 kDa bands on the SDS PAGE, Figures 10 and 11. Amino acid composition data is presented in Table II. The molecular weight calculated from compositional data was 70.0 kDa.

In activity tests on a variety of substrates, specificity was shown for N-acetyl-α-D-galactosamine conjugates. Sugars, other than N-acetyl-α-D-galactosamine were poor substrates, Table III. The mean K_m value for ONP-N-acetyl-α-D-galactosaminide and PNP-N-acetyl-α-D-galactosaminide were 1.58 (s.d. = 0.07, n = 3) and 1.35 (s.d. = 0.01, n = 3), respectively. The enzyme had a broad pH optimum at the range of 6.5 to 7.0, Figure 12. The enzyme was not strongly inhibited by high or low ionic strengths at pH 7.0, Figure 13.

No proteolytic activity was detected in the purified preparations. Aminopeptidase activity was below the limits of detection, <0.005 U mg^{-1} enzyme, with the following substrates: PNA-alanine, PNA-lysine, PNA-leucine, PNA-proline, and PNA-valyl-alanine. In a resorufin-labeled casein protease assay with a sensitivity limit of 0.01 trypsin BAEE units, there was less than 0.01 BAEE U mg^{-1} enzyme detected. This corresponded to less than 0.78 ng of "trypsin-like activity" per mg of pure enzyme. The enzyme

was also tested by an ELISA on human A_2 erythrocyte membranes. Removal of the terminal N-acetyl-α-D-galactosamine residue from the blood group A epitope was achieved by the enzyme as shown in Figure 14.

Discussion

α-N-acetylgalactosaminidase from *Clostridium perfringens* was purified approximately 137-fold and was homogeneous by SDS-PAGE. In a previous report (Levy and Aminoff, 1980), it was hypothesized that the Clostridium enzyme was a multienzyme complex. SDS-PAGE and modular size exclusion chromatography implied that the enzyme in the preparations was monomeric and of lower molecular weight than Levy and Aminoff's estimate. The preparations observed by those authors, contained numerous bands when analyzed by SDS-PAGE. By the present method, the most significant contaminant, neuraminidase, was efficiently removed by the a combination of DEAE Sephadex A-50, SP Sephadex C-50 chromotagraphy, and PBE 94 chromatofocusing steps, the final purification was achieved by HPLC hydroxyapatite chromatography. This is critical and this purification has not been previously achieved. Accordingly, the use of the purified, isolated enzymes was limited. The present invention provides a high activity, isolated and purified enzyme with utility in the area of blood group alteration.

The enzyme was highly specific for the terminal α-N-acetylgalactosamine residues in glycosides, consistent with previous observation (Levy and Aminoff, 1980). The enzyme has no activity for *p*-nitrophenyl glycosides other than *p*-nitrophenyl-α-N-acetylgalactosamine. This is distinct from the achieved spectrum of the homogeneous preparations

from human liver (Dean and Sweeley 1979) and from *Acremonium* sp. (Kadowaki et al, 1989) which exhibited α-N-acetylgalactosaminidase as well as α-galactosidase activity. Hence, the enzyme's utility is recognized for being highly specific in red blood cell type alteratives.

Eucaryotic α-N-acetylgalactosaminidases are lysozomal enzymes and have pH optima in the acidic range of 3.4-4.5 (Dean and Sweeley 1979; Kadowaki et al. 1989; Hata et al. 1992; Sung and Sweeley 1980). The *Clostridium perfringens* α-N-acetylgalactosaminidase shows functional activity in the range of physiological pH. This is an important property in respect to the possible use in enzymatic bioconversion technology, since it allows cell membranes to be modified under physiologic pH conditions. In addition, the Clostridial enzyme can be particularly well-suited to the enzymatic conversion of blood group A to blood group O erythrocytes because the high activity is maintained at close to a neutral pH optima and over a wide range of ion strength, can allow unwashed red cell units to be used. The Clostridial enzyme is also useful for removing α-N-acetyl-D-galactosaminidase from other types of cells expressing the blood group A epitope, for example endothelial cells.

Preparations were free of proteolytic activity which is desirable if cells are to be used for transfusion as numerous erythrocyte antigens can be degraded by exogenous proteases (Wright 1989). Proteolytic modification create the potential for red cell clearance from the circulation. The activity of *Clostridium perfringens* α-N-acetylgalactosaminidase on group A_2 erythrocytes was tested by ELISA. The enzyme efficiently hydrolyzed the terminal N-acetyl-α-galactosamine residues from the blood group A_2 epitope.

Clostridium perfringens α-N-acetylgalactosaminidase can be used for enzymatic conversion of human blood group A_2 red cells to universally transfusable group O red cells. To obtain native enzyme in adequate yield is difficult, therefore, cloning of the α-N-acetylgalactosaminidase gene is used. Cloning can be accomplished by one skilled in the art by using known techniques. A recombinant *Clostridium perfringens* α-N-acetylgalactosaminidase expressed in high yields, is superior to the native lysozomal enzymes for biotechnical applications.

Throughout this application, various publications, including United States patents, are referenced by author and year and patents by number. Full citations for the publications are listed below. The disclosures of these publications and patents in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used is intended to be in the nature of words of description rather than of limitation.

Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the appended claims, the invention can be practiced otherwise than as specifically described.

Table I. Summary of *Clostridium perfringens* α-N-acetylgalactosaminidase purification

Step	Total Volume (ml)	Total protein (mg)	Specific Activity (U/mg)	Total Units (U)	Yield (%)	Fold of Purity
Crude Extract	2250.00	1177.50	0.32	376.92	100.00	1.00
Ammonium Sulfate Precipitation	76.67	204.44	0.53	107.93	28.63	1.65
Sephacryl S-200(I)	483.33	72.50	0.84	60.97	16.18	2.63
DEAE-Sephadex A-50	24.00	14.32	2.92	41.79	11.09	9.12
PBE 94 Chromatofocusing	54.00	1.08	19.73	21.31	5.65	61.65
DEAE-Sephadex A-50	14.00	0.73	20.61	15.10	4.01	64.38
Hydroxyapatite Type I	3.73	0.17	43.92	7.43	1.97	137.20

* Data is expressed as the mean value of three different preparations.

Table II. Amino acid composition of *Clostridium perfringens* α-N-acetylgalactosaminidase

	residues*/mole of protein		residues*/mole of protein
Asp & Asn	51	Tyr	52
Ser	53	Val	33
Glu & Gln	81	Met	16
Gly	51	Lys	42
Arg	28	Ile	55
Thr	28	Leu	51
Ala	32	Phe	46
Pro	17		

*Residue per mole of protein expressed in integer values

Table III. Substrate Specificity of *Clostridium perfringens* α-N-acetylgalactosaminidase

Substrates	Activity (U/ml)
PNP-α-D-galactopyranoside	<0.001
PNP-α-L-arabinopyranoside	<0.001
PNP-β-D-galactopyranoside	<0.001
PNP-N-acetyl-α-D-galactosaminide	0.334
PNP-N-acetyl-β-D-galactosaminide	<0.001
PNP-α-D-glucopyranoside	<0.001
PNP-β-D-glucopyranoside	<0.001
PNP-N-acetyl-α-D-glucosaminide	<0.001
PNP-N-acetyl-β-D-glucosaminide	<0.001
PNP-α-L-fucopyranoside	<0.001
PNP-β-L-fucopyranoside	<0.001
PNP-α-D-mannopyranoside	<0.001
4MU-α-D-galactopyranoside	<0.001
4MU-N-acetyl-α-neuraminic acid	<0.001

* Data is expressed as the mean value of three independent duplicate determination

Table IV. Kinetic Parameters of *Clostridium perfringens* α-N-acetylgalactosaminidase

Substrate	Km
ONP-N-acetyl-α-D-galactosaminidase	1.35
PNP-N-acetyl-α-D-galactosaminidase	1.58

*Data is expressed as the mean value of three independent determinations.

Table 5

1 mkvlgnyiqr nfhydgksfy ttsflnpiln eeilvhtqne fiiyfvdgei

51 lpssemnvei kkqseqllvv nfskdnlsve vnyfvenkvi nkkltvfncc

101 krinyidcdt fefeditnniy ypkkqnnike mgnfngyyve lgqpiyaksl

151 fmgmefpmge nriqerkyfs ryyygksvek rldihsaiig aapekskeki

201 qasffeyika islpatfrkq ynswydhmln itndsiiksf leinrgfkny

251 gitldafvvd dgwanyesvw efndkfpnel kdisecvknl gstlglwigp

301 rggyngtqvt msdwleknkd lnigsknkis ndvnvgdfny lrkmkekmle

351 yqskydisyw kidgmllkpd tedesgpygm htntavyefm islfnelree

401 rgeksfwinl tsyvnpspwf lkwvnslwiq tsqdvgftpn ggndiqkmit

451 yrdsqyyefl ierdiqlplc slynhepiya esasmwyldh qiycsieefk

501 eylmfiatrg nafwefyysy smfdderwev naqaikwiee nypilknstf

551 fgtkpslmgv ygyycqsdsg sksiisfmp sdeiksykle niepkkydvv

601 lgnknykvfe dgsvevklnp keiiilksk

Table 5 continued

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108014                atgaaag tattaggaaa ttatattcaa agaaattttc attatgatgg
108061 aaaaagtttt tataccacat cattttaaa tcctattcta aatgaagaaa tattagttca
108121 tacacaaaat gaatttatta tctatttgt agatggagaa atattacctt cttctgagat
108181 gaatgtggag attaagaagc aaagtgaaca acttttagtg gtgaatttta gtaaagataa
108241 cttatctgtt gaagttaatt attttgtgga aaataaggtt ataaataaaa agctaacagt
108301 tttcaattgt tgtaaacgta ttaattatat tgactgtgat acttttgaat ttgaggatac
108361 taataatatc tattacccta aaaaacagaa taatataaag gaaatgggga attttaacgg
108421 atactatgta gaattagggc aacctattta tgcaaaatct ttattcatgg gaatggaatt
108481 tcctatggga gaaaatcgta ttcaagaaag aaagtatttt tcaaggtatt attatggaaa
108541 aagtgtagaa aaaagattag atatacattc agcaattatt ggagctgctc cagaaaaatc
108601 aaaagaaaaa attcaagctt catttttga gtatattaaa gctatatctt tgccagctac
108661 ttttagaaaa cagtataatt cttggtatga tcatatgcta aacattacta atgatagcat
108721 aataaaaagt ttcttagaaa taaatagagg ctttaaaaac tatggaatta ctttagatgc
108781 ctttgtagtt gatgatggtt gggctaatta tgaaagtgtt tgggaattta atgataagtt
108841 tcctaatgaa ttaaaagata tatcagaatg tgtaaaaaat cttggttcaa ctttaggact
108901 atggattggt ccacgtggtg gatataatgg aactcaagtt actatgagtg attggttaga
108961 aaaaaataag gatttaaaca taggatctaa aaataaaatt tctaatgatg taaatgtagg
109021 agactttaat tatcttagaa agatgaaaga aaaaatgtta gagtaccaaa gcaaatatga
109081 catctcctat tggaaaattg atggaatgtt attaaagcca gatactgagg atgaaagtgg
109141 accatatggt atgcatacta tgacggcagt atatgaattt atgattagtc tatttaatga
109201 gttaagagaa gaaagaggag aaaagagttt ttggatcaat cttacatctt atgttaatcc
109261 tagcccttgg tttttaaagt gggtaaatag tctttggatt cagacttcac aagatgttgg
109321 ctttactcca aatggaggaa atgatattca gaaaatgatc acatatcgtg attctcaata
109381 ttatgaattc ttgattgaaa gagatattca acttccatta tgtagcttat ataatcatga
109441 acctatttat gcagagtctg caagtatgtg gtatttagat catcaaatct attgttctat
109501 agaagagttt aaagagtatt taatgtttat tgctactcgt ggaaatgctt tttgggaatt
109561 ttattattct tattccatgt ttgatgatga acgttgggaa gtaaacgcac aagccattaa
109621 gtggattgag gaaaattatc caatattaaa aaatagtact ttctttggaa caaagcctag
109681 ccttatggga gtatatggat actattgtca atcagattct ggttcaaaat caattatttc
```

Table 5 continued

109741 atttagaaac ccatcagatg aaattaaatc ttataaactt gagaatatag aaccaaagaa
109801 atatgacgta gttctaggca ataaaaatta taaagttttt gaagatggtt ccgttgaagt
109861 taaattaaat cctaaagaaa ttattatact taagagtaaa taa

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